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Spontaneous Erythroid Colony Formation in Brazilian Patients With Sickle Cell Disease

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The ability of circulating progenitor cells to develop erythroid colonies was studied in vitro in the presence or absence of growth factors (5637-CM and erythropoietin) in 63 patients with sickle cell disease (SCD) (36 homozygotes for hemoglobin [Hb] S, 13 double heterozygotes for Hb S and β thalassemia, and 14 SC patients) in Southeast Brazil. In the presence of growth factors, SCD patients (all genotypes) presented significantly higher numbers of circulating burst-forming unit-erythroid (BFU-E/5 × 10^5 MNC), when compared with control subjects. However, when the progenitor cells were cultured in the absence of added stimulus, high numbers of BFU-E were observed only in the genotypes SS and S/β thalassemia. SC patients presented a similar response to the control subjects. Moreover, there was an inverse correlation between spontaneous (without stimulus) BFU-E and Hb levels in SCD patients. These results suggest that the formation of spontaneous BFU-E observed in SCD may be due to an expanded erythropoiesis secondary to hemolysis. Am. J. Hematol. 61:40–45, 1999.

Key words: sickle cell disease; hematopoiesis; BFU-E; autoproliferation

INTRODUCTION

Sickle cell disease (SCD) is a term encompassing a distinct class of hemoglobinopathies. A single point mutation in the sixth position of the β-globin gene, resulting in the substitution of valine for glutamic acid, leads to the replacement of normal hemoglobin (Hb A) by sickle hemoglobin (Hb S). Among these disorders are homozygosity for the Hb S gene or sickle cell anemia (SS), Hb SC disease, or double heterozygosity for the Hb S and Hb C genes, and Hb S/β thalassemia, in which patients are heterozygous for the Hb S and a β-thalassemia gene. A general feature of SCD is its clinical heterogeneity, which seems to be due to associated genetic and environmental modulators [1,2].

SCD patients are characterized by a chronic hemolytic anemia, erythroid hyperplasia in the bone marrow as well as reticulocytosis. They often have increased levels of Hb F, which if sufficiently high may lead to some modulation of clinical symptoms. It is well documented that SS [3–7] and β thalassemic [6,8,9] patients have a higher than normal number of circulating BFU-E. It was demonstrated further that these progenitors expressed hypersensitivity to erythropoietin (Epo) [4,8,10]. In addition, some authors have observed the formation of spontaneous BFU-E colonies (without stimulus) in SS patients [5]. This finding is of particular interest because spontaneous BFU-E colony formation (autoproliferation) is generally thought to be a characteristic feature of myeloproliferative disorders such as polycythemia vera, which is associated with abnormal expansion of erythroid stem cell clones. Despite these well-documented characteristics of erythropoiesis in patients with SS, there have been few studies in SC and S/β thalassemic patients [3]. Based on the above reports and on previous work in our laboratory [11], we designed the present study to investigate the changes in peripheral blood levels of spontaneous and stimulated BFU-E in SS, SC, and S/β thalassemic Brazilian patients as well as in control subjects.

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TABLE I. Hematological Data of Patients With Hemoglobinopathies*

Diagnosis	Age (y.o.)	Hb (g/dl)	MCH (pg)	MCV (fl)	HB A2 (%)	Hb F (%)
SS (n = 36)	27 ± 9	7.8 ± 1.2	30.5 ± 3.8	90.1 ± 9.6	2.3 ± 0.5	6.4 ± 4.0
Sβ (n = 13)	28 ± 13	8.9 ± 1.3	22.4 ± 2.3	68.4 ± 7.3	3.7 ± 0.6	7.7 ± 4.3
SC (n = 14)	32 ± 11	10.9 ± 1.3	25.7 ± 2.8	79.6 ± 7.4	—	1.6 ± 0.7

*Hb, hemoglobin; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume. Values are expressed as mean ± SD. Hematologic indexes (MCH, MCV) and Hb levels were obtained by the Celldyn 1600 CS Counter (Abbott, Unipath/Mountain View, CA).

SUBJECTS AND METHODS

Patients

Sixty-three patients with hereditary hemoglobinopathies (36 SS, 13 Sβ, and 14 SC) were studied. They had not received transfusion for the last 4 months. All were clinically stable and the diagnosis was based on clinical, familial, and laboratory data, including electrophoresis on cellulose acetate at pH 8.9 and on agar gel at pH 6.2, solubility test, estimation of Hb F and Hb A2 [12], and in most cases, family studies [13]. All the patients gave informed consent and the study was approved by the Ethical Committee of this hospital. Thirty normal individuals were used as control. Clinical and hematological data of these patients are presented in Table I.

Peripheral Blood Cells Separation

Mononuclear cells were separated from 10 mL of heparinized peripheral blood by 30 min centrifugation at 400*g* in Ficoll-Hypaque (density, 1.077 g/mL) (Pharmacia Fine Chemicals AB, Uppsala, Sweden). The cells from the interface were washed three times with RPMI 1640 (Sigma Chemical Company, St. Louis, MO) and enumerated for BFU-E culture.

Assay for 14-day BFU-E

Assays with mononuclear cell suspensions were performed in 2 mL agar cultures in 35 mm Petri dishes using 5×10^5 cells/culture. The medium used was Iscove's modified Dulbecco's medium (Sigma) containing 20% fetal calf serum (Sigma) and 0.6% agar. Colony formation was studied with or without addition of the conditioned medium derived from the human bladder carcinoma cell line 5637 and human recombinant Epo (2 IU/mL) (Amersham International, Bucks, England).

The plates were incubated at 37°C in 5% CO_2 in air at 100% humidity. Colonies were counted at 35× magnification using a dissection microscope [14].

Statistical Analysis

Kruskal-Wallis test was used to examine the presence of difference in the BFU-E response in patients with Hb SS, Hb S/β thal, Hb SC, and controls. Dunn's procedure was performed to determine which groups were different. Associations between variables were evaluated based on the nonparametric Spearman's rank correlation.

RESULTS

The individual values for the growth and differentiation of early circulating erythroid progenitors (BFU-E/5 $\times 10^5$ MNC) in the presence of 5637-CM plus Epo in the 63 SCD patients are presented in Figure 1. We observed an increased number of BFU-E in SCD patients (all genotypes) in relation to controls ($P < 0.001$). However, when the progenitors cells were cultured in the absence of exogenous stimulating factors (Fig. 2), high numbers of BFU-E were found only in the genotypes SS and S/β thalassemia ($P < 0.001$). SC patients presented significantly lower numbers of spontaneous BFU-E colonies when compared with SS patients, showing a similar response to the control subjects.

Spontaneous BFU-E presented an inverse correlation with Hb levels in all SCD patients studied (Fig. 3) ($r_s = -0.4$, $P = 0.00058$). This negative correlation did not hold for each of the phenotypes separately. The levels of Hb F presented no correlation to BFU-E colonies.

DISCUSSION

The data presented here demonstrate that the number of circulating BFU-E is increased in SCD patients, despite the genotype SS, SC, or S/β thalassemia. This increased response was also observed in SS and S/β thalassemic patients when the progenitor cells were cultured in the absence of added stimulus. SC patients presented significantly lower numbers of spontaneous BFU-E colonies when compared with SS patients, showing a similar response to the control subjects. These findings are in agreement with data from the literature, which report that the hematologic parameters in the genotype SC are generally less abnormal than in the SS [15,16]. Moreover, we found an inverse correlation between spontaneous

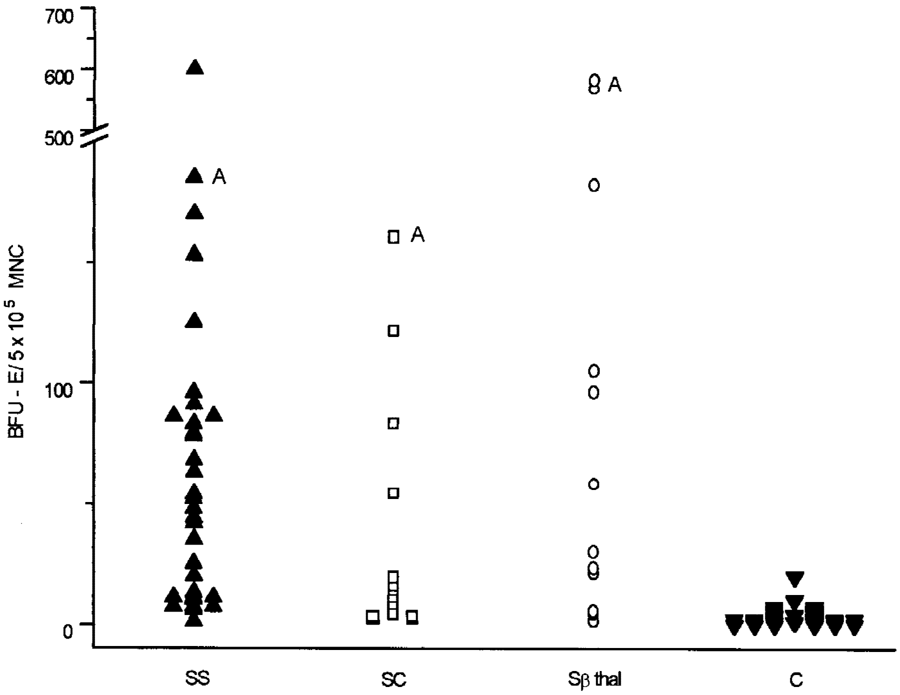

Fig. 1. Numbers of early circulating erythroid progenitor cells (BFU-E/5 × 10^5 MNC) in SCD patients [SS (▲), (n = 36); SC (□), (n = 14); Sβ (○), (n = 13)] and 30 control subjects (▼). Progenitor cells were cultured in the presence of 5637 CM stimulating factor plus Epo. *P* < 0.001.

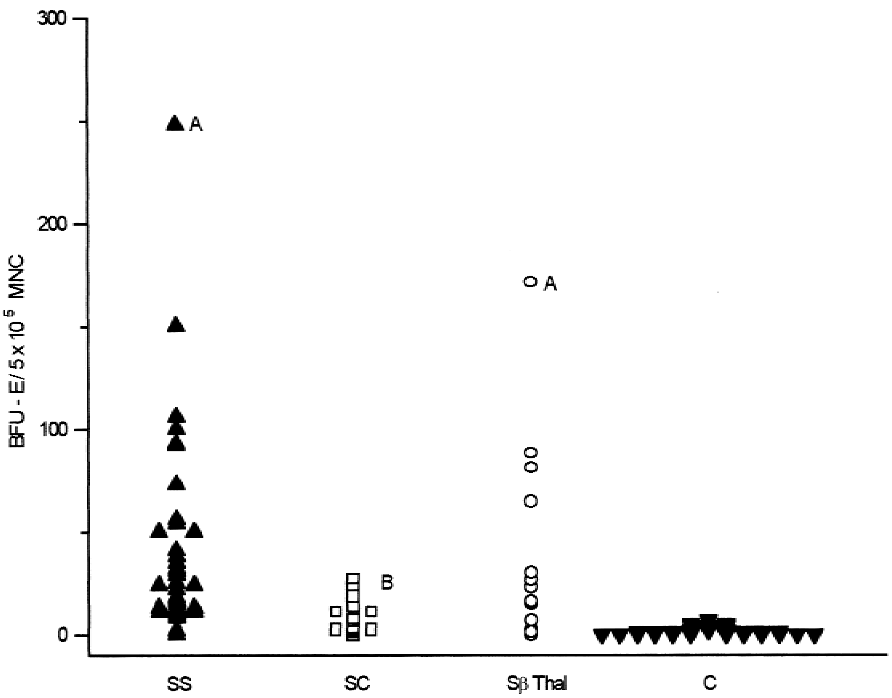

Fig. 2. Numbers of early circulating erythroid progenitor cells (BFU-E/5 × 10^5 MNC) in SCD patients [SS (▲), (n = 36); SC (□), (n = 14); Sβ (○), (n = 13)] and 30 control subjects (▼). Progenitor cells were cultured in the absence of added colony-stimulating factors (autoproliferation). *P* < 0.001. A, significant in relation to controls.

BFU-E and Hb levels, which presumably reflects the expanded erythropoiesis secondary to hemolysis in hemoglobinopathies.

As mentioned before, such spontaneous BFU-E colony formation is considered typical of myeloproliferative disorders, such as polycythemia vera [17–24] and primary thrombocythemia [19,22–25]. The only common feature in all these diseases that could explain this similarity is the increased sensitivity to Epo [4,6,10,18,20,24]. Epo hypersensitivity in SS as well as β thalassemic patients has been demonstrated by several authors [4,5,8,10] using Epo dose-response curves. These findings demonstrate considerable colony formation at low Epo concentrations, when compared with control subjects. In this regard, Pennathur-Das et al. [10] suggested that Epo hypersensitivity could be associated with chronic erythroid hyperplasia. Although no reports were found in the literature in relation to other hemolytic anemias, our results

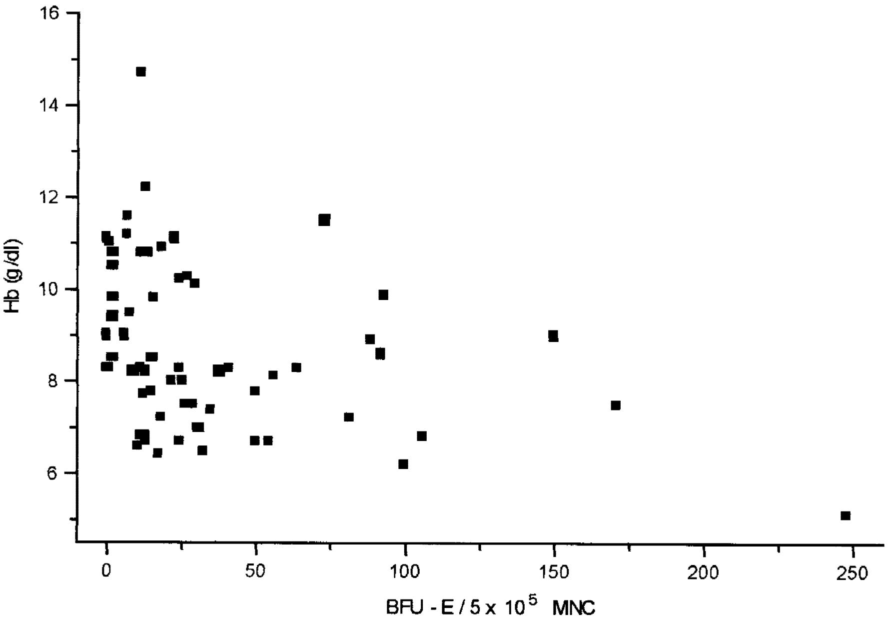

Fig. 3. Correlation between the number of nonstimulated BFU-E/5 × 10^5 MNC and the Hb levels in all patients with SCD studied. ($r_s = -0.4$, *P* = 0.00058.) Negative correlation did not hold for each of the phenotypes separately. A, significant in relation to controls; B, significant in relation to SS.

corroborate this hypothesis because SS and S/β thalassemic patients showed an increased formation of spontaneous BFU-E, whereas SC patients, who have less hemolysis and more restricted bone marrow expansion, did not present this response.

It has been suggested previously that the number and proliferation of BFU-E are not influenced by anemia or hypoxia, states characterized by elevated Epo levels [26,27]. However, in a mouse model, Axelrad et al. [28] reported that bone marrow BFU-E may be recruited to proliferate under conditions of major hematopoietic stress produced by repeated bleeding. Hence, high levels of circulating Epo might have expanded the number of erythroid progenitors in SCD before the sample was obtained for culture. Although we have not measured Epo levels in these patients, unexpectedly low serum Epo (sEpo) levels have been found previously in patients with SS [29]. This finding, however, does not exclude the mechanism proposed above, because low sEpo levels may be related to a faster clearance of Epo in erythroid hyperplastic states. Moreover, a recent report [30] points to an inverse relationship between red blood cell precursor mass and sEpo levels. The most likely explanation for this is that sEpo levels are regulated not only by the rate of renal production, but also by the rate of utilization by erythroid cells.

Some authors have suggested that the production of spontaneous BFU-E–derived colonies in myeloproliferative disorders may be associated with the presence of fetal calf serum (FCS) in the culture medium, which normally contains biologically active levels of Epo [17,25,31]. This hypothesis was supported by Casadevall et al. [32], who demonstrated that endogenous colonies were present in serum-containing medium and absent when serum was omitted from the cultures in polycythemia vera. In other serum-free culture systems [19,33], however, a similar erythropoietic response was observed under both conditions. Conflicting observations in different serum-free culture systems are not surprising because serum-free media often contains biological materials, such as albumin, which can be contaminated with factors capable of modulating erythropoiesis. In this regard, the recent development of a truly serum-free culture system [34], which uses a fatty acid-free and globulin-free preparation of albumin instead of the ordinarily used albumin preparation, is of particular relevance to accurately dissect the complex effects of hematopoietic growth factors in the modulation of normal and abnormal hematopoiesis. Thus, it will be interesting to extend our studies of spontaneous erythroid colony formation in SCD using this serum-free system.

It is well known that the molecular integrity and action of Epo receptors are of key importance for the kinetics of red cell production. Thus, it is possible that this Epo hypersensitivity may occur as a result of changes in the erythroid progenitors such as increased numbers of Epo receptors, increased accessibility of otherwise cryptic receptors, activation or change in the affinity of the receptors for the ligand on the surface of the progenitor cells, and/or altered Epo signal transduction pathway [5,35–39]. In this regard, previous work from our laboratory [11] has shown as increased expression of Epo receptors in SCD patients, which presented a high correlation with both stimulated and endogenous BFU-E. This possibility is consistent with recent studies, which show that defects in the Epo receptor may account for certain disease states, such as familial erythrocytosis and polycythemia vera [40–43].

Further investigation in patients with other types of hemolytic anemia is necessary to establish whether the formation of endogenous BFU-E is a common occurrence in all states of erythroid hyperplasia.

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